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Dose-response relationships in chemical carcinogenesis: renal mesenchymal tumours induced in the rat by single dose dimethylnitrosamine

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Summary. A single intraperitoneal dose of dimethylnitrosamine (DMN) given to weanling rats after 3 days' treatment with a protein-free diet results in the induction of renal mesenchymal tumours, the incidence of which is related to the dose of DMN in a sigmoid dose-response curve.

The number of tumours per kidney is small, most animals given 40mg/kg DMN (the TD_{100}) having either one or two tumours in each kidney. However, within a few days of dosing a large number of small proliferative foci of mesenchymal cells, which resemble the tumours, appears in the renal cortex. The number of these foci is linearly related to the dose of DMN. By 12 weeks, the majority of these foci have disappeared, leaving an essentially normal kidney with only one or two developing tumours. The initial amount of methylation of guanine at the O^6 and 7 positions in the kidney DNA measured 18 h after dosing is also linearly related to the dose of DMN. Thus, the formation of the early lesions is directly proportional to the amount of DNA alkylation, but the eventual tumour incidence is not. It is suggested that the mechanisms which operate to remove the majority of the early proliferative foci determine the shape of the dose-response curve for the tumours, which is independent of the initial alkylation levels.

Keywords: dimethylnitrosamine, renal mesenchymal tumours, preneoplastic lesions, dose-response relationship, methylation, threshold

Toxicologists and regulatory authorities rely on the dose-response relationships for chemicals in animals to provide the basis for extrapolation downwards to define a 'safe' dose and upwards to estimate a dose level potentially toxic to man. The prediction of risk at given exposure levels requires extensive information about the dose-response relationship, particularly at low levels of exposure, and in most studies this information is inadequate. An attempt was made to tackle this problem in the massive ED_{01} study (ED_{01} Task Force 1981), but the data obtained proved to be very difficult to interpret and much controversy has arisen over the choice of a valid mathematical model for the dose-response relationship.

Dose-response relationships for carcinogens present in the diet and environment are of great significance to man, who is simulta-

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neously exposed to low levels of many chemicals which have been demonstrated to be carcinogens in experimental systems. Examples are the nitrosamines, which are both ingested and formed in the gut, and known tumour promoters such as phenobarbitone and alcohol (Driver & McLean 1986). The interaction of several sets of dose-response relationships will determine the eventual tumour incidence.

In order to study dose-response relationships for carcinogens at very low levels, enormous numbers of animals are required and the problems encountered in the ED_{01} study remain unsolved. Another approach to the problem is to study the mechanisms which underly the nature of the dose-response relationship.

In order to do this, a very simple model of carcinogenesis was chosen for the present study, in which a single dose of a complete carcinogen is able to induce up to 100% incidence of a morphologically well-defined tumour at a dose which is not lethal to the animal if a dietary pre-treatment is used. Rats are fed on sucrose for 3 days before a single intraperitoneal dose of dimethylnitrosamine (DMN) as originally described by Swann and McLean (1968). Renal mesenchymal tumours start to appear at 12–16 weeks (Hard & Butler 1970*a*). Before the development of recognizable tumours, small proliferative foci of mesenchymal cells are seen in the renal cortex which are likely to represent early or pre-neoplasia (Hard & Butler 1970*b*). The dose-response characteristics for these early lesions and for the final tumours have been investigated. Alkylation of the kidney DNA has also been measured. As renal mesenchymal tumours never occur spontaneously in the rat, there was no problem of a background rate, which was one of the confounding factors in the ED_{01} study. However, the identification of the early lesions did present a problem. Although they resemble the ultimate tumour with respect to their cellular morphology, many are extremely small and therefore difficult to find and count. Two special staining methods were employed to facilitate their identification: mucin secreted by the cells stained positively with alcian blue and very soon after treatment with DMN, the enzyme guanidinobenzoatase was expressed on the cell surface, which could be identified by a fluorescent marker. This paper presents data for the dose-response curves for the mesenchymal tumours at 20–24 months, for the early proliferative lesions at 3 weeks, and for initial levels of DNA methylation 2 h and 18 h after dosing with dimethylnitrosamine.

Materials and methods

Male Fischer F344 rats bred at the MRC Laboratories were weaned and immediately put onto a diet of pure sucrose for 3 days.

DMN obtained from Sigma made up in normal saline was given by a single intraperitoneal injection of between 2 mg/kg and 50 mg/kg. The rats were then returned to the pelleted MRC Diet 41B for the duration of the experiment.

Control animals were also fed on sucrose and then given a single intraperitoneal injection of sterile normal saline.

All animals received tap water *ad libitum* throughout the experiments.

Experiment 1. Dose-response curve for tumours. Groups of rats were given 2, 5, 10, 15, 20, 25, 30, 40 or 50 mg/kg DMN. They were killed when moribund or at 24 months. The number of rats per group was chosen in order to provide more data at the critical lower doses (Table 1).

Experiment 2. Time course for development of early lesions. Groups of 20 rats were given 40 mg/kg DMN (the ED_{100}) and killed after 1, 3, 6, 8, 10, 12, or 16 weeks. Parallel control groups of 20 rats were given saline and killed at the same time.

Experiment 3. Dose-response curve for early lesions. Groups of 20 rats were given 2, 5, 10, 20 or 40 mg/kg DMN and killed after 3

Table 1. Dose-response relationship for renal tumours after single dose DMN

DMN (mg/kg)	*n*	Mesenchymal tumours in: 2 or 1 (percentage of animals)	2	1	0 kidneys	Epithelial tumours in 1 or 2 kidneys
50	12	100	75	25	0	25
40	14	100	66	34	0	28
30	10	100	50	50	0	30
25	16	100	19	81	0	12
20	8	87	50	37	13	50
15	25	32	4	28	68	12
10	68	3	0	3	97	0
5	80	1	0	1	99	0
2	38	0	0	0	100	0
0	50	0	0	0	100	0

Animals killed at 20–24 months. Pretreated with 3 days on sucrose. Single i.p. injection of DMN in saline.

weeks. Twenty control rats were given saline and also killed at 3 weeks.

Experiment 4. Alkylation of kidney DNA by dimethylnitrosamine. Rats were given *N,N*-[^{14}C]dimethylnitrosamine (Specific activity 58 mCi/mmol, 12 μCi/rat in 0.14 M NaCl intraperitoneally) diluted with unlabelled material to give doses of from 2 to 40 mg/kg as indicated. Animals were killed either 2h or 18 h after dosing. The livers and kidneys were removed, frozen in liquid nitrogen and stored at -70°C. Extraction of the DNA from the kidneys pooled from five rats was according to the procedure of Kirby as modified by Swann and Magee (1968). Analysis of the purine bases from DNA was achieved following mild acid hydrolysis (0.1 M HCl for 30 min at 70°C). Unlabelled markers, 7-methylguanine (Sigma) and O^6-methylguanine (a gift from Dr P.F. Swann, London) were added to the hydrolysate. The pH was adjusted to 6.8 (using M-NH_4OH) and the samples (4 ml) were chromatographed on a Sephadex G-10 column (75 × 1.5cm) using 0.1 M ammonium formate buffer, pH 6.8 as eluant (Rabes *et al.* 1979). Fractions (7 ml) were collected and analysed for their absorption at 260 nm. Radioactivity was determined in a Searle liquid scintillation counter following the addition of scintillant. Conversion of c.p.m. to d.p.m. was computed using the external standard ratio method.

Histology. All rats were killed by CO_2 inhalation and full necropsies were performed. The kidneys, livers and lungs were removed for histology and also any organ with a visible abnormality. Lungs were inflated with buffered formalin via the trachea. All organs were then immerse-fixed in buffered formalin.

Paraffin-embedded sections were cut and stained with Harris' haematoxylin and eosin. In addition the kidneys were stained with alcian blue with a neutral red counterstain.

A third serial section of each kidney was de-waxed and stained with 9-aminoacridine followed by propidium iodide and examined under fluorescence according to the technique of Steven *et al.* (1985) to identify cells expressing guanidinobenzoatase.

Results

The percentage of animals bearing mesenchymal and/or epithelial tumours in the kidney at the end of 20–24 months is shown in Table 1. Because weanling animals were used, the number of epithelial tumours was small compared with the mesenchymal type (Hard 1979). In this system a threshold exists at 15 mg/kg, below which no epithelial tumours were seen, despite quite large groups ($n=68$ at 10 mg/kg, $n=80$ at 5 mg/kg). Above this threshold, the percentage of animals bearing epithelial tumours was unrelated to dose.

The percentages of animals with mesenchymal tumours either in both kidneys or in only one, are shown in Fig. 1. There is a clear dose-response relationship for the number of tumours induced per animal, as well as for the number of animals affected. At 25 mg/kg, 32% of animals have renal mesenchymal tumours, but in almost all the animals, only one kidney is involved. Usually only one tumour was seen. At 30 mg/kg, all the animals had mesenchymal tumours, half of them with tumours in both kidneys, whilst at 50 mg/kg, 75% of animals had tumour in both kidneys. Because the tumours were often very large (10 g was not uncommon), it was usually difficult to ascertain how many tumours were present in each kidney at the end of 24 months. Shorter term experiments show that there are very rarely more than two discrete tumours per kidney at 40 mg/kg DMN.

Histologically the tumours showed the same range of morphological types as those described by Hard and Butler (1970*a*). The majority were partially cystic, the predominant cell being spindle shaped, forming areas of dense cellularity, frequently with a storiform pattern (Fig. 2). Normal structures, such as tubules and glomeruli, were surrounded by invading tumour and remained well preserved at the periphery (Fig. 3). The most common variant was the appearance of haemangiomatous areas. A small proportion (10%) of the tumours had invaded beyond the renal capsule, but no distant metastases were seen. All the tumours contained some areas of sparse cellularity with alcian blue-positive mucin surrounding the spindle cells (Fig. 4). All the tumours were strongly positive for guanidinobenzoatase, the enzyme being expressed by the spindle cells and also by any pre-existing tubules which had become surrounded by tumour. Adjacent areas of kidney were negative. In control kidneys only lymphocytes were positive for guanidinobenzoatase.

Figure 5 shows the dose-response relationship for the mesenchymal tumours pooling

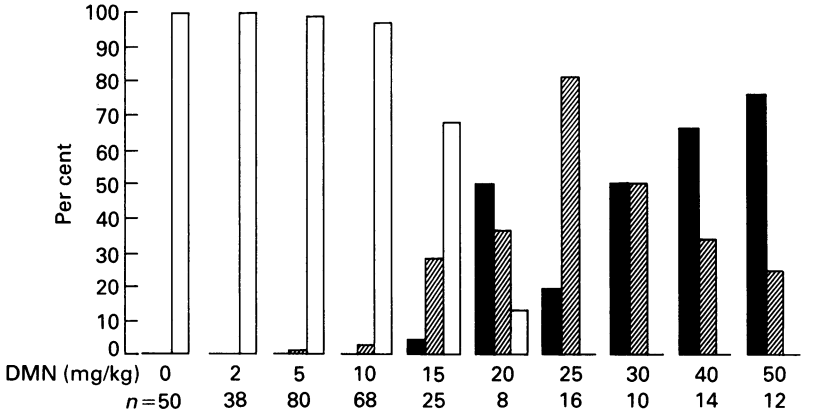

Fig. 1. Incidence of renal mesenchymal tumours at 20–24 months after DMN ■, Per cent animals with tumour in two kidneys; ▨, per cent of animals with tumour in one kidney; □, per cent of animals with tumour in neither kidney.

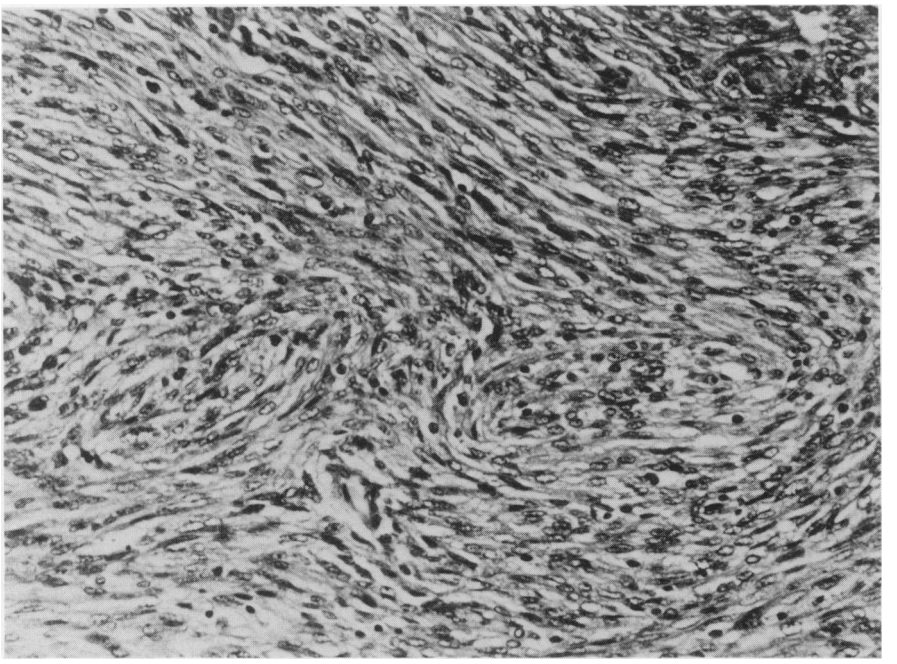

Fig. 2. Mesenchymal tumour 24 months after DMN. The cells are predominantly spindle shaped and are densely packed in a storiform pattern. H & E, × 100.

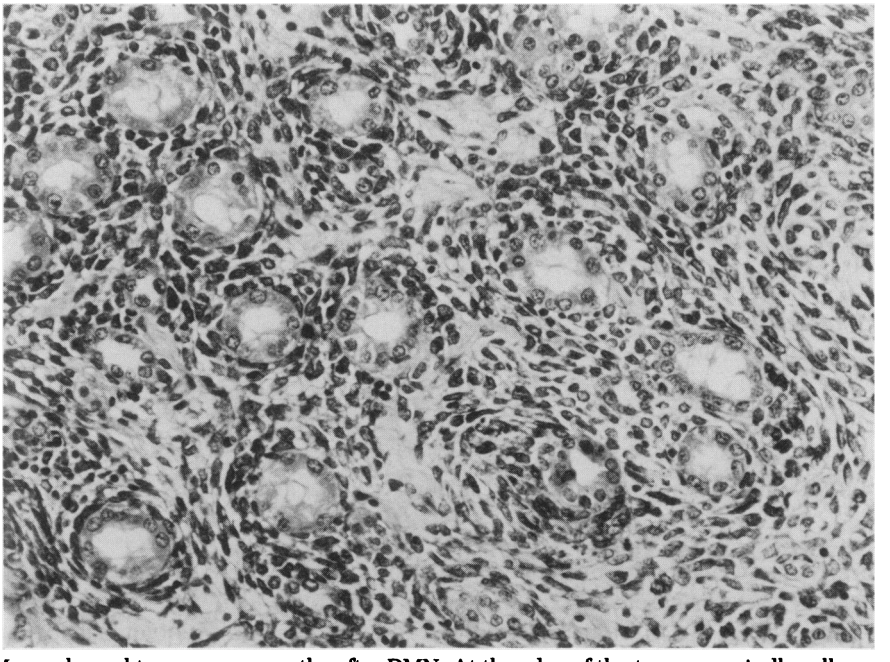

Fig. 3. Mesenchymal tumour 24 months after DMN. At the edge of the tumour, spindle cells are aligned around pre-existing renal tubules. H & E, × 100.

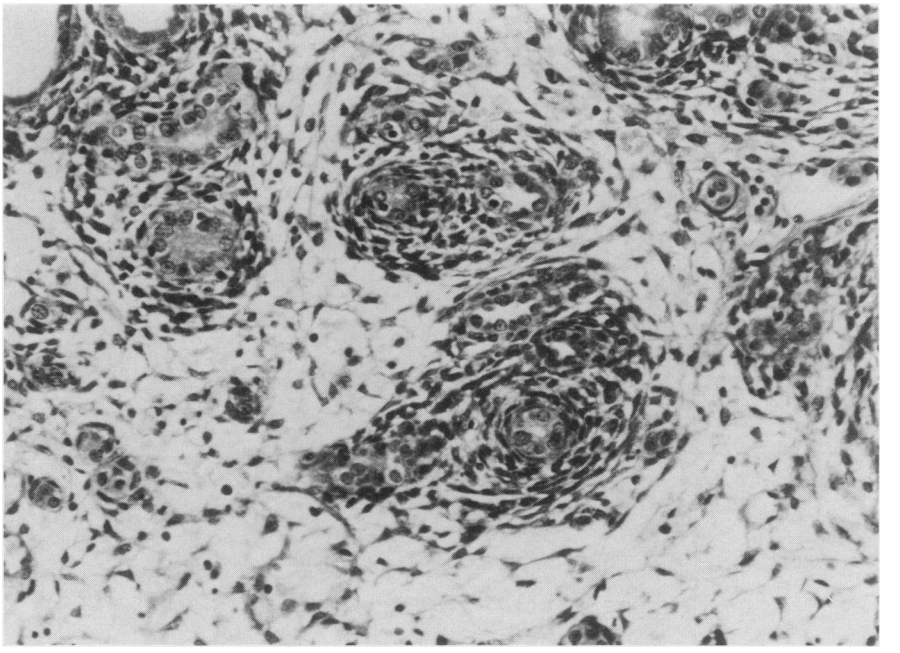

Fig. 4. Mesenchymal tumour 24 months after DMN. As in Fig. 3, tumour cells are aligned around pre-existing tubules, but there are also areas of sparse cellularity. These areas are composed of mucin, which stains positively with alcian blue.

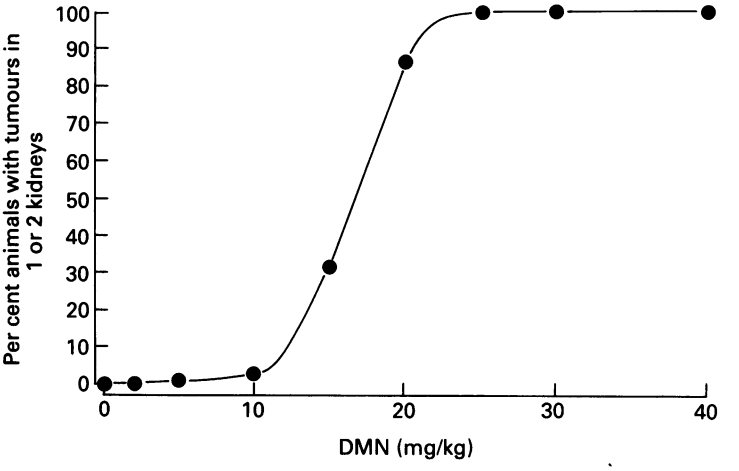

Fig. 5. DMN-induced renal mesenchymal tumours at 20–24 months: dose response relationship.

the figures for one or two kidneys affected. This shows a classical sigmoid shape with an ED_{50} of 17.5 mg/kg, the curve rising steeply between 10 and 20 mg/kg.

Figure 6 shows the dose-response relationship for the early lesions at 3 weeks. These proliferative foci were identified by a combination of their morphological appearance on haematoxylin and eosin paraffin sections and their staining characteristics with alcian

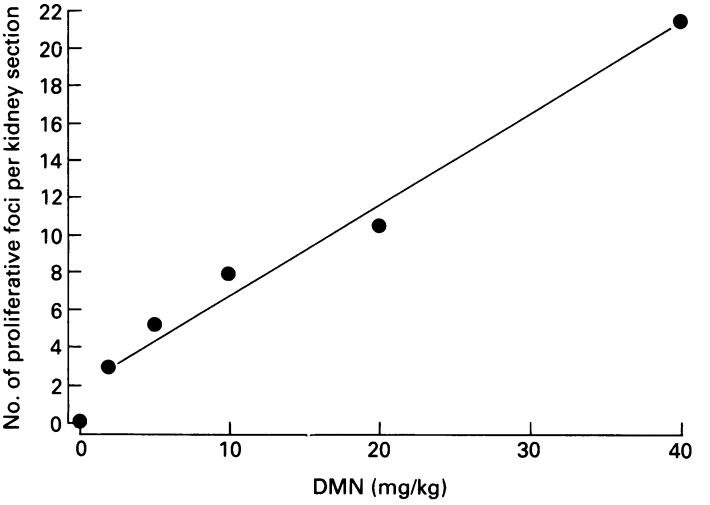

Fig. 6. DMN-induced mesenchymal proliferative foci at 3 weeks: dose-response relationship.

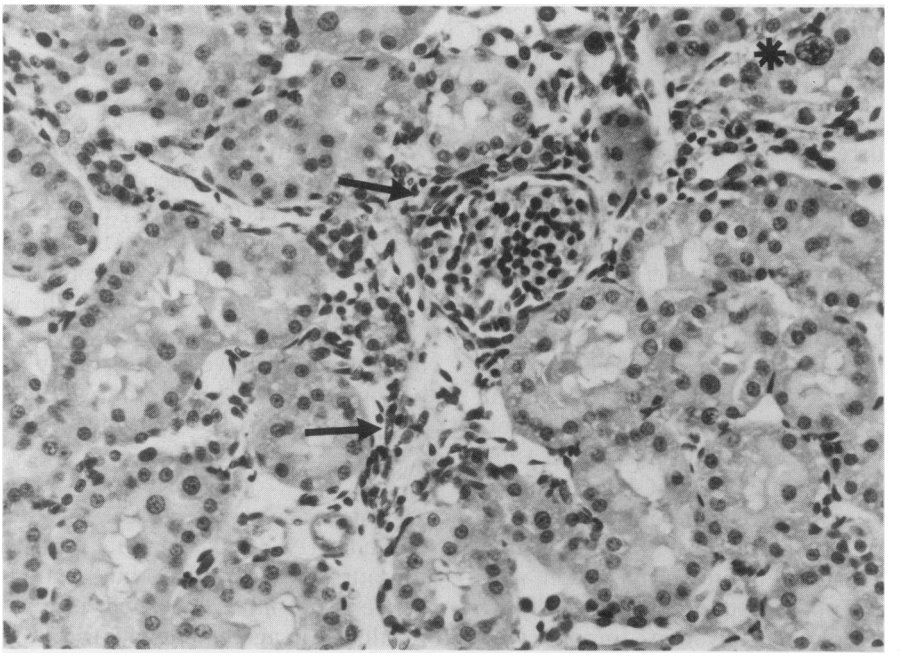

Fig. 7. A small proliferative focus 3 weeks after DMN, adjacent to a glomerulus. Many of the cells in the focus are spindle shaped (→) and are surrounded by mucin as in Fig. 4. Note the pleomorphism of the tubular epithelial cells, with a few bizarre nuclei (*). H & E, × 100.

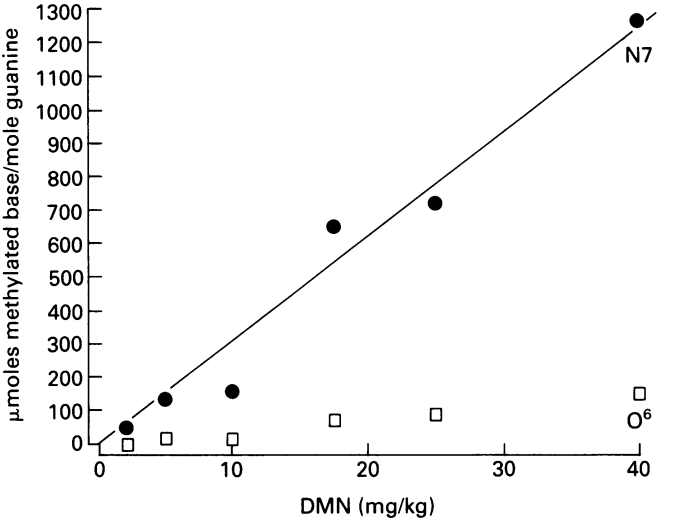

Fig. 8. Methylation of kidney DNA by DMN: dose response relationship.

blue and for guanidinobenzoatase. Their appearance altered with time, as described by Hard and Butler (1970*b*). At 3 weeks, they were maximal in number and consisted of an aggregation of spindle cells with a few lymphoid cells, usually in association with a glomerulus or a small arteriole (Fig. 7). They were scattered throughout the renal cortex, but occurred more frequently in the outer zone. All were highly positive for guanidinobenzoatase and many contained small amounts of alcian blue-positive material. The number of these foci was counted in a single, complete longitudinal section through each kidney to give an estimate of the total number. Before 3 weeks, the lesions were frequently diffuse and therefore difficult to quantify, but thereafter became more discrete. By 10 weeks most of the foci had disappeared, leaving areas of sparse cellularity in the cortex, containing a few lymphocytes and fibroblasts. By 12 weeks, small tumours had begun to form, which were sometimes up to 1 cm in diameter by 16 weeks. Morphologically, these tumours were identical to the more cellular areas of the tumours seen at 24 months. They were alcian blue positive and expressed guanidinobenzoatase. Control kidneys showed no alcian blue positive material at any dose or time. Only lymphocytes and macrophages were positive for guanidinobenzoatase.

The dose-response curve for the early proliferative foci counted at 3 weeks is linear from 2 to 40 mg/kg DMN with the number of early lesions given by $2.36 + 0.464 \times$ dose. The methylation of kidney DNA by DMN is also linear with respect to dose. Figure 8 shows the levels of O^6 and 7 methylguanine, measured 18 h after the DMN. At 2 h after the DMN the levels were only slightly higher and were also linear with dose. The levels of O^6 and 7 methylguanine were also measured in the livers and were consistently found to be about six times higher than in the kidney.

Discussion

The sigmoidal shape of the dose-response curve for DMN-induced renal mesenchymal tumours demonstrates that tumour incidence is not linearly related to dose. These results are in agreement with those of Swann *et al.* (1980), although their experiments did not investigate the lower end of the dose-response curve. Peto *et al.* (1984) have

carried out an extensive dose-response study on several different nitrosamines in rats, mice and hamsters. Using an enormous number of animals, they were able to calculate the 'Weibull *b*-values' for oesophageal and liver tumours. The present results are compatible with those of Peto *et al.* (1984), but are not directly comparable as there seem to be considerable differences between organs. In our experiments, only 1% of the animals given 5 mg/kg DMN developed a tumour by 24 months and no tumour were induced by the lowest dose of 2 mg/kg, suggesting that there may be an effective threshold. A threshold also appears to exist for the renal epithelial tumours. The dose-response characteristics for these tumours are much less clear, probably because the numbers are relatively small as the model is designed to maximise the number of mesenchymal relative to epithelial tumours.

Within 24 h of dosing with DMN, at 40 mg/kg, extensive changes occur in the epithelial cells of the renal cortex. There is some necrosis of the tubular cells, which is rapidly followed by hypertrophy and hyperplasia. Most of these changes regress by 12 weeks, but a few tubules remain hyperplastic and may give rise to adenomata. One small adenoma was seen at 16 weeks.

Small proliferative foci of mesenchymal cells appear in the kidney cortex quite rapidly after dosing with DMN (Hard & Butler 1970*b*). The morphological similarity between the spindle cells present in these foci and those of the mesenchymal tumours suggests that they may represent early neoplastic or preneoplastic lesions, which may be capable of progressing to form tumours. In order to facilitate counting these small lesions, two stains were employed. Alcian blue stains the mucin which is almost always present in the mesenchymal tumours and it was found that the early lesions also contained small amounts of alcian blue-positive material. It was often possible to distinguish a few spindle cells stained with neutral red against a background of alcian blue positive mucin. Guanidinobenzoatase is a proteolytic enzyme which was first described in association with mouse Ehrlich ascites tumour cells (Steven & Al-Ahmad 1983). Its physiological role is unknown, but it has been demonstrated in many other tumour cell types and also in various normal tissues which are 'active'; for example, secretory tissues such as pancreas and the lactating breast are positive. Inflammatory cells, such as lymphocytes and macrophages also express guanidinobenzoatase. The normal rat kidney is negative for guanidinobenzoatase except for that seen in the inflammatory cells. All the tumours examined were positive for guanidinobenzoatase, so this enzyme provided a convenient marker for the early lesions. Any lesion which could be identified on the H & E or alcian blue stained sections was positive for guanidinobenzoatase. In addition, the presence of the enzyme in interstitial cells sometimes drew attention to small foci which had been missed using the other techniques. Serial sections stained with H & E, alcian blue, and with 9-aminoacridine and propidium iodide were therefore prepared for every kidney.

Using these techniques, the numbers of early lesions were obtained at different times and found to be maximal at 3 weeks. The the dose-response curve for the early lesions was therefore obtained at this time and was found to be linear between 2 mg/kg and 40 mg/kg DMN. Even at 2 mg/kg, which induces no tumours, a significant number of early proliferative foci were seen. The number of early lesions is therefore directly proportional to the amount of DNA methylation, which is also linearly related to dose. No threshold exists for either methylation of DNA or the formation of the small proliferative foci. However, the bulk of these foci disappear, leaving only one or two developing tumours per kidney by 12–16weeks. It is the mechanism which operates to remove these foci and not the initial amount of binding to the DNA, which therefore determines the shape of the final tumour dose-response curve. The levels of O^6 and 7 methylguanine in the kidney DNA were measured at 2 h and 18 h

after DMN. Such alkylation does not of course distinguish between that occurring in mesenchymal and other cell types. At all doses of DMN the levels were only slightly lower when measured at 18 h. It has been shown (Nicoll *et al.* 1975) that O^6 methylguanine is much longer-lived in the kidney than in the liver. However, we consistently found the levels of 7 and O^6 methylguanine in the liver to be about six times those found in the kidney. No liver tumours were seen in these experiments, suggesting that either a different threshold of alkylation required for carcinogenesis exists for the two organs or that other factors are responsible for the tumour yield.

The mechanism which operates in the kidney to remove the majority of the early proliferative foci is of great interest. It seems likely that the system is analogous to that seen in the rat liver in which a large number of hyperplastic nodules of various staining characteristics develop following treatment with an initiator and promoter. Only a small sub-population of these nodules develops into hepatocellular carcinoma (Bannasch *et al.* 1985; Peraino *et al.* 1986). In the kidney, most of the early lesions disappear, either by spontaneous regression or as a result of host defence mechanisms. The prominence of an inflammatory reaction from about 3 to 10 weeks after the DMN suggests that immune surveillance may play a role. By 12 weeks, most of the lesions have disappeared, leaving a few areas of sparse cellularity and only one or two developing tumours.

It is generally accepted in toxicology that the extent of any adverse effect is directly related to the level of exposure to the chemical, there being a threshold dose, below which no detectable adverse effects occur. However, in regulatory carcinogenesis, there is a desire to ignore this relationship and to define a chemical as a carcinogen or a non-carcinogen, irrespective of dose. This would seem to be in conflict with accepted toxicological precepts in that it assumes that there is no threshold for carcinogenesis, even though it is apparent that thresholds do exist for other forms of toxicity. Clearly, if one assumes that the incidence of cancer is simply proportional to the amount of DNA alkylation, no threshold can exist and therefore there is no 'safe' dose. This is the assumption which underlies the Delaney Clause (Coulston 1979). However, these experiments demonstrate that in this model there is a critical event occurring long after the alkylation of the target organ DNA has taken place, which determines the tumour yield. Thus, although alkylation takes place even at very low doses, an effective threshold may exist for carcinogenesis which is independent of this initial alkylation level. Knowledge of the mechanisms which underly the dose-response relationships for carcinogens may permit more informed regulation of their use.

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